Plant Science 179 (2010) 267–272

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Physiological and molecular changes during opening and senescence of *Nicotiana mutabilis* flowers

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ARTICLE INFO

Article history:
Received 17 March 2010
Received in revised form 24 May 2010
Accepted 26 May 2010
Available online 4 June 2010

Keywords:
Anthocyanin
Ethylene
Fragrance
Petal
SAG12 (senescence-associated gene)
Senescence

ABSTRACT

As the flowers of *Nicotiana mutabilis* open and senesce, their petals show a striking color change from white through pink to red. This was associated with an increase in chalcone synthase (*CHS*) gene expression and a substantial rise in petal anthocyanin content. It was also accompanied by up-regulation of 1-aminocyclopropane-1-carboxylic acid oxidase (*ACO*) transcripts and elevated rates of ethylene production at the onset of petal wilting. Emission of the fragrant monoterpenoid volatiles 1,8-cineole, linalool, and terpineol also increased as petals developed pink coloration. The increase in volatile emission was preceded by a rise in monoterpene synthase (*MTS*) gene expression. Transcripts of a homologue of *SAG12*, a senescence-associated gene encoding a cysteine protease, began to accumulate in petals 3 days prior to visible wilting. Exposure of newly opened white flowers to 1 $\mu L L^{-1}$ ethylene accelerated petal coloration, wilting, and induction of *SAG12* expression by ca. 1 day while treatment with 500 $nL L^{-1}$ 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, retarded these processes. The numerous genetic and experimental tools available for tobacco can readily be applied to this close relative, which therefore provides an interesting new model for studying ethylene-mediated flower senescence.

1. Introduction

Flower senescence is a complex metabolic process under strict control by developmental and environmental signals [1]. Visible as wilting and/or fading of floral organs, senescence is preceded and accompanied by extensive degradation of nucleic acids, proteins and membranes [2]. It is also associated with considerable changes in gene transcription and translation [3]. For example, numerous genes were found to display dramatic up- or down-regulation expression patterns during iris and alstroemeria petal senescence [4,5].

A number of senescence-associated genes (SAGs) encode catabolic enzymes such as proteases and nucleases [3]. Proteases regulate metabolic and developmental processes by facilitating turnover of rate-limiting proteins [6]. Cysteine proteases appear to play a particularly important role in senescence as they are consistently identified in senescing floral tissues [7–9]. Among the cysteine proteases, *SAG12* has been most closely related to senescence. Transcripts of *SAG12* are only detected at the onset of visible senescence in Arabidopsis leaves [10,11] and tobacco flowers [12]. Thus, *SAG12* may represent a useful and specific marker for the study of floral senescence.

The phytohormone ethylene is the primary regulator of floral senescence in a wide range of plant genera [13]. In sensitive species, petal senescence is associated with an increase in endogenous ethylene production, while exposure to exogenous ethylene greatly accelerates the process. Moreover, treatment with inhibitors of ethylene biosynthesis and action delay flower senescence [14]. Studies with species such as carnation, daffodil and petunia have revealed that ethylene coordinates and enhances expression of several floral senescence-associated cysteine proteases [7,15,16].

A close relative of tobacco, *Nicotiana mutabilis*, was recently discovered in southern Brazil [17]. As its specific epithetic suggests, the petals of *N. mutabilis* change in color from white through pink to red as the flowers age. Although changes in color are associated with floral senescence in a range of other species (*Cichorium intybus*, *Lupinus perennis*, *Desmodium setigerum*, *Gossypium hirsutum*) these species are not as nearly well-studied at the molecular level as species of tobacco. Emission of fragrant volatiles, namely 1,8-cineole and linalool, were also found to increase by 2–9-fold as *N. mutabilis* petals developed pink coloration [18]. Pollination of flowers was reported to reduce their longevity [19], suggesting that ethylene may regulate flower senescence. By virtue of its close relationship to the model plant tobacco, a range of genetic tools would also be available for the study of *N. mutabilis* flowers. Accordingly, *N. mutabilis* potentially represents an interesting alternative system for molecular studies of ethylene-mediated flower senescence.

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doi:10.1016/j.plantsci.2010.05.011

Fig. 1. Photographs of individual *Nicotiana mutabilis* flowers and the typical change in flower opening and petal color over 7 days in planta. Scale bar represents 1 cm.

We characterized physiological and molecular changes during opening and senescence of *N. mutabilis* flowers. Our results illustrate the temporal and developmental relationships among petal opening, coloration, volatile production, and the onset of senescence, as determined by the expression of a senescence-associated *SAG12* homologue (*NmSAG12*). We also evaluated the role of ethylene in these events.

2. Materials and methods

2.1. Plant material

N. mutabilis Stehmann & Semir plants were grown from seed in the greenhouse at the University of California, Davis at 28/18 °C (day/night) from March to September using standard cultural practices. Flower buds opened with white petals that changed to light pink and then through to dark pink and red as the flower aged during a 7-day lifespan (Fig. 1). Each day of development was nominally assigned as a different stage between opening (Day 0) and senescence (Day 6). Flowers were tagged on Day 0 (flowers starting to open). The tagged flowers were then harvested daily at different developmental stages for experiments evaluating changes in petal coloration and gene expression. For all other experiments (i.e. ethylene production, respiration, and the effects of ethylene), flowers were harvested at Day 0 and maintained in deionized water under the evaluation conditions noted below.

2.2. RNA isolation and cDNA preparation

Flowers at each of the seven stages of opening and senescence were harvested on three separate occasions. Petals were excised and immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from a 1 g aliquot of frozen petals pooled from 5 to 7 flowers using TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's directions. Isolated RNA was treated with Turbo DNA-free™ DNase (Ambion Inc., Austin, TX, USA) to remove contaminating genomic DNA. The RNA concentration was determined using a spectrophotometer (DU 800; Beckman Coulter Inc., Fullerton, CA, USA) at 260 nm, and its integrity was verified by electrophoresis on 1% (w/v) agarose in 1× TAE (Tris-Acetate-EDTA) buffer (Fisher Scientific Inc., Fair Lawn, NJ, USA). First-strand cDNA was then synthesized from 2 μg total RNA, oligo d(T) primer and random hexamers using SuperScript® III reverse transcriptase (Invitrogen Corp.) in accordance with the manufacturer's instructions.

2.3. Primer design

The PCR primers for selected biosynthetic genes for ethylene (1-aminocyclopropane-1-carboxylic acid oxidase, *ACO*), pigment (chalcone synthase, *CHS*) and fragrant volatiles (monoterpene synthase, *MTS*), and of a homologue of *SAG12* are shown in Table 1. Primers were designed from conserved regions of nucleotide sequences from members of the Solanaceae (petunia, tobacco, tomato) on the GenBank database using the BLAST program (National Center for Biotechnology Information, NCBI). The MTS sequence displayed 89% homology at the protein level to a biochemically characterized *Nicotiana suaveolens* 1,8-cineole synthase [20]. The abundance of tobacco 18S rRNA served as an internal control (Table 1). These primers were used in PCR assays to quantify the abundance of gene transcripts in the different flower stages.

2.4. Semi-quantitative RT-PCR and real-time quantitative qRT-PCR

cDNA from each flower opening and senescence stage was added to a PCR master mix containing primers for each gene under study plus taq DNA polymerase and buffer (Invitrogen Corp.) as prescribed by the manufacturer. For RT-PCR, samples were denatured at 94 °C for 5 min followed by 25–40 sequential cycles of 30 s each at 94, 55 and 72 °C and one final cycle at 72 °C for 5 min using a thermo cycler (model 2720; Applied Biosystems, Foster City, CA, USA). PCR products were separated by electrophoresis on 1% (w/v) agarose in 1× TAE buffer containing 0.1 $\mu L\,mL^{-1}$ SYBR® Safe DNA gel stain (Invitrogen Corp.). Gene expression data were verified by quantitative real-time qRT-PCR analysis, using SYBR® green PCR master mix (Applied Biosystems) and a 7300 real-time PCR system (Applied Biosystems). Reaction conditions were as described above for RT-PCR. Data were analyzed using the $2^{-\Delta\Delta CT}$ method [21] and are presented as the relative level of gene expression [22].

2.5. PCR product identification

DNA bands corresponding to *ACO*, *CHS*, *MTS* and *SAG12* homologues were extracted from agarose gels using a QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA). The extracted DNA was ligated into pGEM®-T Easy vectors (Promega Corp., Madison, WI, USA) and transformed into 5-α F′ I^q competent *Escherichia coli* cells (New England BioLabs Inc., Ipswich, MA, USA) as directed by the manufacturers. Plasmid DNAs were then isolated from the bacterial host using a QIAprep® Spin Miniprep Kit (Qiagen Inc.). The inserts were sequenced using T7 sequence primer at the DNA Sequencing Facility at the University of California, Davis. Sequences were compared against existing GenBank entries using BLAST (NCBI). The partial sequences of mRNAs for *N. mutabilis ACO*, *CHS*, *MTS* and *SAG12* are deposited in GenBank as accession numbers FJ590433, FJ590432, FJ620661 and FJ590434, respectively.

Table 1
Primers used for PCR of target genes in *Nicotiana mutabilis* petals.

Gene	Forward primer	Reverse primer
ACO	5′-TTTTACAATCCAGGAAGTGATGC-3′	5′-ATCTTGGCTCCTTAGCTTGAAAC-3′
CHS	5′-TCATGATGTACCAACAAGGTTG-3′	5′-ACAACAGTCTCAACTGTAAGCC-3′
MTS	5′-AGGCTTGGAGTTAGTTACCACTT-3′	5′-TAAGTAAGCTTTGCACAAATCTG-3′
SAG12	5′-AGTGGCTAATCAACCTGTTTCG-3′	5′-ATGCGCATATATCCACTGTCAC-3′
Nt18S rRNA	5′-CATGGCCGTTCTTAGTTGGTGGAG-3′	5′-AAGAAGCTGGCCGCGAAGGGATAC-3′

2.6. Ethylene and 1-methylcyclopropene (1-MCP) treatment

Flowers were harvested upon opening (Day 0) and their pedicels were placed into microcentrifuge tubes containing deionized water. They were sealed into glass chambers and treated with either 1 μL L^{-1} ethylene or 500 nL L^{-1} 1-MCP (released from EthylBloc®, Floralife Inc., Walterboro, SC, USA) for 12 h at 20 °C as previously described by Serek et al. [23]. Additional flowers held in air served as controls. Five replicate flowers from each treatment were then maintained for evaluation at 20 °C, 50% relative humidity and 18 μmol m^{-2} s^{-1} light (12 h/day) until the onset of petal wilting at the petal margins.

2.7. Ethylene production and respiration

Flowers were harvested on the day of opening (Day 0) and maintained in deionized water as described above during their opening and senescence. Eight flowers were sealed individually into plastic 55 mL capacity vials every 8 h. After a 4-h incubation, the concentration of ethylene and CO_2 that accumulated in the headspace inside vials was determined using a gas chromatograph (model 111; Carle Instruments Inc., Anaheim, CA, USA) fitted with a photoionization detector (model P1-51; HNU Systems Inc., Newton, MA, USA), and an infrared gas analyzer (model PIR-2000R; Horiba Instruments Co., Irvine, CA, USA), respectively. Ethylene and CO_2 concentrations were determined against a calibration curve obtained with known standards (Airgas Inc., Sacramento, CA, USA).

2.8. Anthocyanin content

Petals were excised from five replicate flowers at each opening and senescence stage described above and frozen in liquid nitrogen. Frozen tissues (100 mg) were ground to a powder, extracted in 1 mL 1% (v/v) HCl acid (37.6% ACS grade, Fischer Scientific Inc.) in 99% methanol (99.8% grade, EMS Science, Gibbstown, NJ, USA) and held overnight at 4 °C. The absorbance of the supernatant was determined at 535 nm using a spectrophotometer (Beckman Coulter Inc.). The total anthocyanin concentration was then calculated by comparison to absorbance data of serial dilutions of a cyanidin-3-glucoside (Sigma–Aldrich Inc., St Louis, MO, USA) standard.

2.9. Collection of floral volatiles

Eight replicate flowers were harvested daily at 1800 h at each opening and senescence stage. Individual flowers were placed into deionized water plus 5% (w/v) sucrose (Fisher Scientific Inc.) and enclosed into 900 mL glass desiccators for 12 h at 20 °C in darkness. Volatiles were collected during the night when maximum emission from *N. mutabilis* is typically observed [18]. Vacuum pumps (model DC12/16FK; Furgut GmbH, Tannheim, Germany) circulated headspace air in a closed loop at 2 L min^{-1} through borosilicate glass columns (3 mm i.d.) packed with 25 mg Porapak™ Q (80–100 mesh; Supelco, St Louis, MO, USA). Trapped volatile compounds were eluted from columns into Teflon-capped glass vials with 250 μL dichloromethane (HPLC grade 99.9%, Fisher Scientific Inc.). A 20 μL aliquot of 1.1 mM 2-methylbutylisovalerate (SAFC Supply Solutions®, St Louis, MO, USA) was added to each sample and served as an internal standard. Samples were held at −80 °C pending analysis.

2.10. Analysis of floral volatiles

Volatile samples (2 μL) were injected in splitless mode into an Agilent 6980N gas chromatograph (GC) coupled to an Agilent 5975B mass spectrometer (MS) (Agilent Technologies Inc., Santa Clara, CA, USA). Compounds were separated in a HP-5MS-3 (5% phenylmethyl siloxane) non-polar capillary column (30 m long × 0.25 mm i.d. × 0.25 μm film thickness; Agilent Technologies Inc.) using ultra high purity helium (Praxair Inc., Sacramento, CA, USA) as the carrier gas. Column temperature was held at 60 °C for 3 min and then increased at 10 °C min^{-1} to 260 °C, where it was maintained for 7 min. The GC–MS transfer line, ion source and quadrupole mass analyzer were operated at 250 °C, 230 °C and 150 °C, respectively. Ionization energy was set at 70 eV. The MS scanned from 40 to 300 amu. Compounds were tentatively identified using a computerized mass spectral reference library (NIST MS Search 2.0, National Institute of Standards and Technology, Gaithersburg, MD, USA). The sum total of emitted compounds was expressed as a proportion of the internal standard on the basis of peak area. The concentration of the three most abundant compounds (1,8-cineole, linalool, terpineol) was then determined by comparison to serial dilutions of authentic standards.

3. Results

3.1. Changes in pigmentation and ethylene, CO_2 and fragrance production

The development of pink and red coloration of *N. mutabilis* petals during the later stages of flower opening and senescence (Fig. 1) was strongly correlated with a substantial increase in the anthocyanin content of petals (Fig. 2A). 1,8-Cineole, linalool and terpineol were the major components of *N. mutabilis* floral scent and collectively accounted for 39–57% of total volatile production. Emission of these terpenoid compounds increased by 3–26-fold during flower opening and reached a maximum on Day 3 in association with the start of anthocyanin accumulation in the petals (Fig. 2B). Thereafter, rates of emission decreased to basal levels as flowers wilted (Fig. 2B). A similar pattern in the emission of total volatile compounds was also observed (data not shown). Within 2 days of opening, rates of ethylene production by detached flowers increased 2.4-fold (Fig. 3A) and remained at this elevated rate through the onset of flower senescence. This increase in ethylene production occurred as flowers developed a mid-pink coloration (data not shown). Freshly harvested flowers had a high respiration

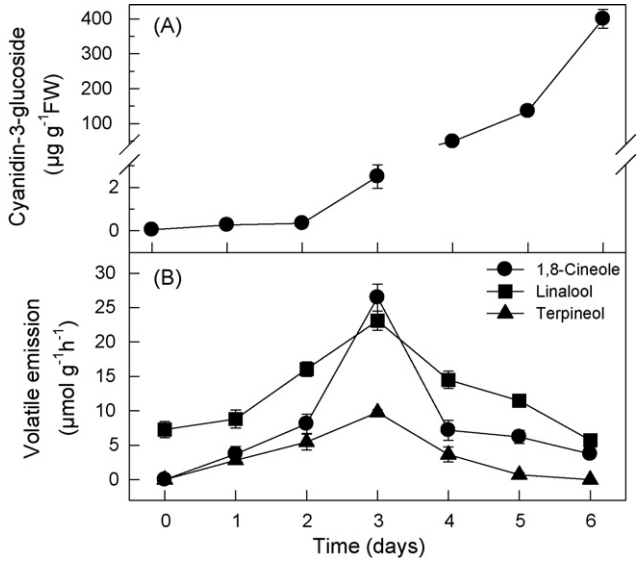

Fig. 2. Change in anthocyanin content in petals (A), and fragrance volatile emission (B) from detached flowers of *Nicotiana mutabilis* at sequential stages of flower opening and senescence over 7 days. Data represent means ± s.e.m. (n = 8). Where no error bars are present, the s.e.m. was smaller than the size of the symbol.

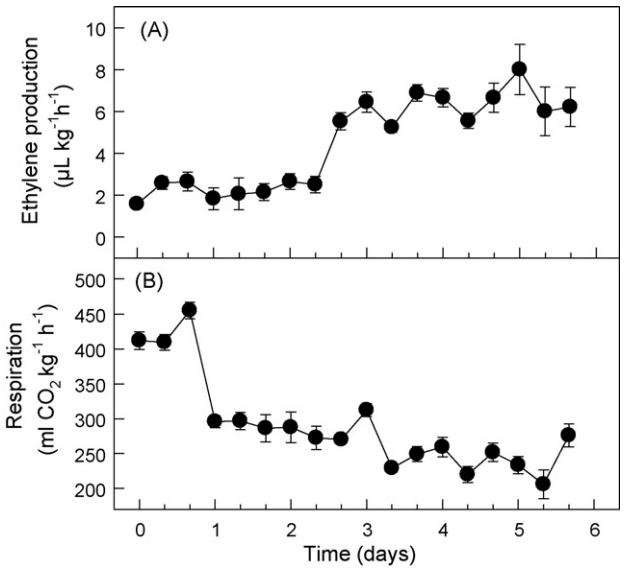

Fig. 3. Change in ethylene production (A), and respiration (B) from detached newly opened flowers of *Nicotiana mutabilis* in tubes of water during their opening and senescence over 7 days. Data represent means ± s.e.m. ($n = 8$). Where no error bars are present, the s.e.m. was smaller than the size of the symbol.

rate, which fell substantially on Day 1, and then gradually during the remainder of their postharvest life (Fig. 3B). There was no climacteric rise in ethylene production and respiration.

3.2. Gene expression during flower opening and senescence

ACO transcripts were detected in petals at all seven stages of flower opening and senescence (Fig. 4). The abundance of *ACO* mRNA increased nearly 30-fold between Days 0 and 3 before decreasing to low levels by Day 6 (Fig. 4). The genes encoding the flavonoid and putative monoterpenoid biosynthesis enzymes, *CHS* and *MTS*, respectively, were also expressed in all seven flower stages (Fig. 4). *CHS* transcript levels were highest in Day 2 flowers that were beginning to change color and thereafter decreased as petal coloration continued to develop (Figs. 1 and 4). Similarly, *MTS* expression levels in flowers increased to a maximum on Day 2 before declining over time (Fig. 4), and this pattern preceded similar changes in volatile emission rates by flowers by 1 day (Fig. 2B). Abundance of transcripts of a *SAG12* homologue increased 11-fold between stages 2 and 3 as petals developed a mid-pink coloration and displayed maximum *ACO* transcript accumulation (Figs. 1 and 4). *SAG12* expression further increased as flowers continued to age and was nearly 600 times as strong on Day 6 as on Day 0.

3.3. Ethylene effects on petal color and senescence

Exposure to 1 μL L^{-1} ethylene for 12 h at 20 °C reduced the postharvest life of newly opened flowers by 1 day (Fig. 5), by accelerating the development of visible symptoms of petal senescence (wilting). Treatment with ethylene also hastened the increase in petal anthocyanin content (Fig. 6). Transcripts of *SAG12* were detectable after just 1 day from the commencement of ethylene treatment in comparison to 2 days for the controls (Fig. 7). In this particular experiment, the display life of detached flowers was shorter than that of flowers left attached to the plant (3 days vs 7 days). Pre-treatment of flowers with 500 nL L^{-1} 1-MCP for 12 h at 20 °C extended flower display life by 3 days relative to control flowers (Fig. 5), and delayed the associated accumulation of anthocyanin in petals (Fig. 6). 1-MCP treatment also delayed the expression of *SAG12* transcripts in petals until Day 4 of postharvest life (Fig. 7).

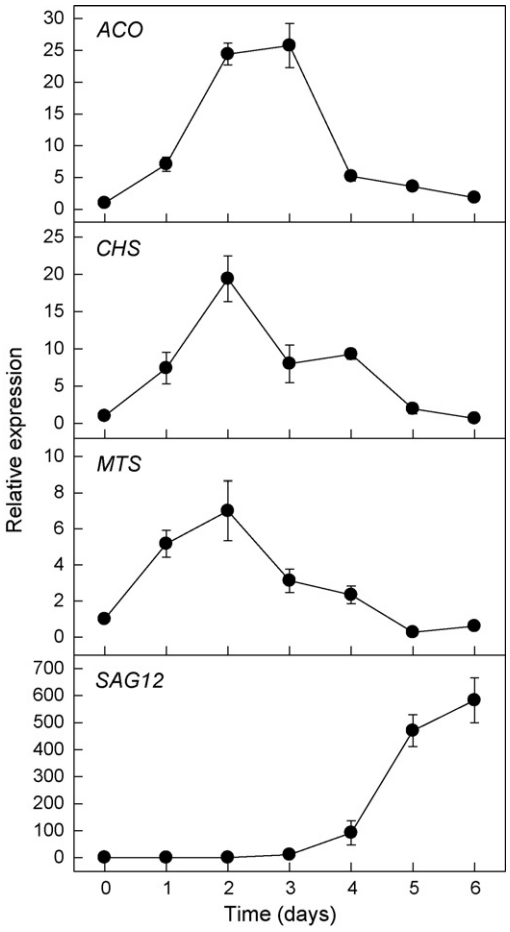

Fig. 4. Expression patterns of *ACO*, *CHS*, *MTS* and *SAG12* transcripts in the petals of *Nicotiana mutabilis* at sequential stages of flower opening and senescence over 7 days. Abundances were determined by comparison with an internal rRNA 18S control and are shown relative to the expression level on Day 0, which was given a value of 1. Data represent means ± s.e.m. ($n = 9$). Where no error bars are present, the s.e.m. was smaller than the size of the symbol.

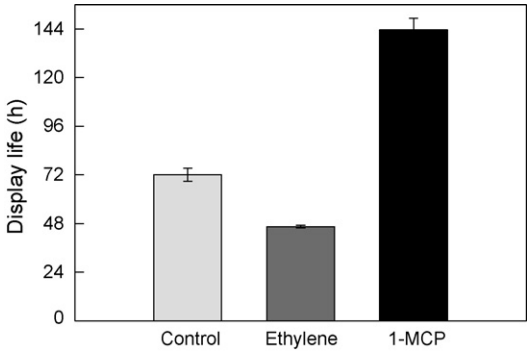

Fig. 5. Display life at 20 °C of detached newly opened flowers of *Nicotiana mutabilis* in tubes of water following treatment with 1 μL L^{-1} ethylene or 500 nL L^{-1} 1-MCP for 12 h at 20 °C. Flowers held in air served as the control. Data represent means ± s.e.m. ($n = 5$).

4. Discussion

N. mutabilis plants produce numerous flowers that are typically short-lived (3–7 days). Pollination of *N. mutabilis* flowers has been

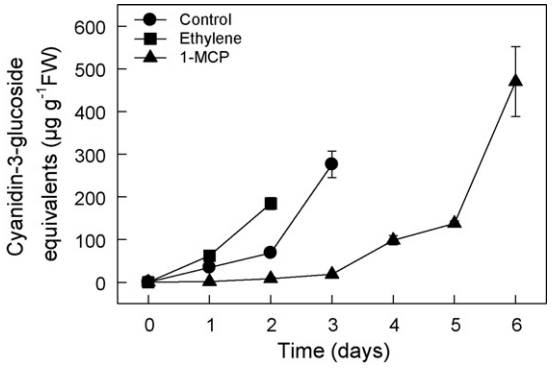

Fig. 6. Changes in the anthocyanin content in petals of detached newly opened flowers of *Nicotiana mutabilis* following treatment with 1 μL L^{-1} ethylene or 500 nL L^{-1} 1-MCP for 12 h at 20 °C on Day 0. Flowers held in air served as the control. All flowers were maintained in tubes of water at 20 °C until petals wilted. Data represent means ± s.e.m. (n = 5). Where no error bars are present, the s.e.m. was smaller than the size of the symbol.

reported to reduce flower longevity [19] implicating ethylene as a regulator of senescence [24]. Petal wilting was correlated with elevated rates of ACO gene expression and ethylene production (Figs. 1, 3A and 4), while exposure to exogenous ethylene accelerated the onset of senescence (Fig. 5). Moreover, pre-treatment with 1-MCP, an ethylene binding inhibitor, extended the life of detached flowers (Fig. 5). While increased rates of ethylene biosynthesis were associated with flower senescence, there was no distinct climacteric rise in ethylene production or respiration.

A particularly interesting feature of *N. mutabilis* petals is their striking color change from white through pink and ultimately to red by the onset of senescence. This change is associated with an increase in anthocyanin content of the petals (Figs. 1 and 2A), and preceded by an increase in *CHS* expression (Fig. 4). In other tobacco species (*N. sylvestris*, *N. tabacum*, *N. tomentosiformis*) in which petals develop pink coloration prior to flower opening, *CHS* is also expressed relatively early in development [25]. Because *CHS* acts early in the flavonoid and anthocyanin biosynthesis pathway, downstream genes such as anthocyanidin synthase may be more important determinants of anthocyanin accumulation [26]. Increased rates of ethylene production by *N. mutabilis* flowers also accompanied the change in petal pigmentation (Fig. 3A). Moreover, exogenous ethylene accelerated petal coloration and anthocyanin content (Fig. 6) while treatment with the anti-ethylene action agent, 1-MCP, retarded the process. Although color changes are

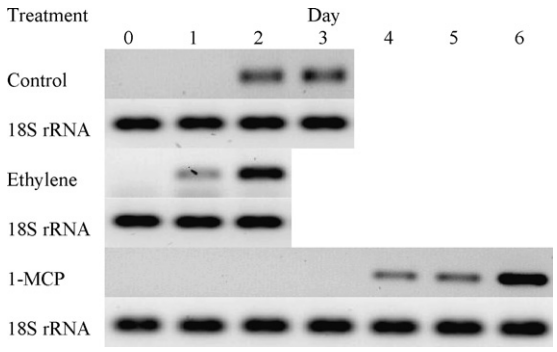

Fig. 7. Photographs of 1% agarose gels showing expression of *SAG12* transcripts isolated from *Nicotiana mutabilis* petals on each day of postharvest life at 20 °C. Newly opened flowers were treated with 0 (control) or 1 μL L^{-1} ethylene or 500 nL L^{-1} 1-MCP for 12 h at 20 °C on Day 0 and then maintained in tubes of water at 20 °C until petals wilted. The abundance of 18S rRNA served as an internal control. Data are representative of three biological replicates.

often seen during floral aging, the involvement of ethylene in the regulation of this process has only been reported in a few species such as *Lupinus*, *Cymbidium* and *Viola* [27–29].

The development of *N. mutabilis* flowers was also accompanied by a substantial increase in emission of the volatile monoterpenoids 1,8-cineole, linalool and terpineol (Fig. 2B) as previously reported [18]. We also show that emission of these volatiles decreases as flowers senesce, and this suggests that combined changes in olfactory and visual cues may discourage visits from hummingbirds, the principle pollinator of *N. mutabilis*, as the flowers senesce [17]. We found that *MTS* transcript abundance peaked 1 day ahead of maximum volatile production (Figs. 2B and 4), suggesting that emission is regulated at the level of gene expression [30]. The decline in volatile emission after Day 3 was preceded by an accumulation of *ACO* transcripts (Fig. 4). Ethylene has been reported to mediate the pollination-induced reduction in scent production by petunia flowers [31], while exogenous ethylene accelerates loss of fragrance in sweet pea flowers [32]. While exogenous ethylene reduced floral longevity in *N. mutabilis* (Fig. 5), we did not assess whether this treatment also reduced scent production.

Cysteine proteases have been reported to be up-regulated during petal senescence in a range of species including alstroemeria [9], carnation [7], daffodil [8], daylily [33], iris [34] and sandersonia [35]. One such cysteine protease, *SAG12*, is considered to represent one of the best molecular markers for senescence, as it is detected in Arabidopsis leaves and tobacco flowers [10–12] only when they are visibly senescing. In *N. mutabilis*, *SAG12* transcripts began to accumulate along with a marked change in petal color, 2 days prior to visible flower senescence (wilting) (Figs. 1 and 4). *SAG12* transcript abundance continued to increase until the flowers wilted.

Exposure to ethylene has been reported to hasten the induction of a range of senescence-associated cysteine protease genes (*pDCCP1*, *PhCP2*, *PhCP8*, *PhCP9*, *PhCP10*, *DAFSAG2*) in carnation, daffodil and petunia petals [7,36,16]. In our study of *N. mutabilis*, we show that exogenous ethylene can also advance the onset of *SAG12* up-regulation in petals, while treatment with 1-MCP delayed its expression and associated flower senescence (Fig. 7). Ethylene similarly enhances *SAG12* expression in Arabidopsis leaves, but only in older and visibly yellowing foliage [11]. Our data are in general agreement with an earlier observation that a *SAG12:GUS* transgene was strongly expressed in tobacco flowers after pollination, a process generally associated with elevated rates of endogenous ethylene production [12].

Identification of model species such as carnation [37], daffodil [16], four o'clock [38] and petunia [15] has contributed greatly to our understanding of the role of ethylene in stimulating flower senescence. *N. mutabilis* may be a very useful additional system for the study of ethylene-mediated flower senescence. Owing to its close taxonomic relationship to tobacco and other members of the Solanaceae (e.g. petunia), a number of well established genetic and experimental tools (e.g. expressed sequence tags, transformation protocols, virus-induced gene silencing) could readily be applied to *N. mutabilis*. Furthermore, the striking petal color change that accompanies *N. mutabilis* flower opening and senescence represents a unique system to study the mediating role of ethylene in floral senescence and associated petal pigment and volatile biosynthesis. This pronounced petal coloration may also lend itself to aid the identification of senescence-delaying genes in *N. mutabilis* petals through a newly devised protocol known as TASSEL (transposon-associated senescence-specific enhancer-linked) tagging (S. Gan, personal communication). These tools may also be used to study relationships between petal pigment and fragrance volatile biosynthesis. The novel features of ethylene-mediated floral senescence in *N. mutabilis* whereby petals change color and emit fragrant volatiles prior to an increase in *SAG12* expression highlights interesting opportunities for further study of this species.

Acknowledgements

We thank the American Floral Endowment and the USDA Floriculture Initiative for partial funding of this study. We also acknowledge Antonio Ferrante and Setal Prabhu for technical assistance with experiments.

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